CHEMICAL VERIFICATION OF THE C1q RECEPTOR SITE ON IgG

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1. Introduction

Antibody molecules are bifunctional: the recognition of foreign molecules by the Fab regions triggers the interaction of a variety of effector systems which are mediated through the Fc region, some 75A from the antigen recognition site. Activation of the effector systems culminates in the elimination of these foreign molecules. It has been more than 10 years since the positive identification of the antibody combining site by sequence analysis [1,2] followed by chemical modification and peptide mapping studies [3]. Concurrently with the elucidation of the molecular sequence [4] and three-dimensional structure of the immunoglobulin molecule [5–7] there have been continual efforts to locate the site of the many immunoglobulin effector functions. The best characterized of these effector systems is the classical complement system [8,9]. Porter and colleagues unambiguously identified the Cγ2 domain as the domain which binds C1q, the first subcomponent of complement [10]. Efforts to elucidate the molecular details of the site triggering the complement effector system by: (1) inhibitory polypeptides [11–14]; (2) chemical modifications of antibodies [12,15,16]; (3) analysis of IgG sequences [17] have proved ambiguous and controversial. In [18] we proposed a receptor site on the Cγ2 domain of IgG for the C1q molecule based on 3 lines of analysis: (1) conservation of sequence and accessibility of amino acid residues to solvent; (2) inhibitors of the C1q–IgG interaction; (3) chemical modification of amino acid residues on IgG and C1q. The proposed site was on the two C-terminal β-strands of the Cγ2 domain which contains several highly conserved and accessible Glu, Lys, Thr and Ser residues (fig.4b). This postulate was consistent with the results of inhibitor studies and chemical modifications which suggested that: (1) C1q–IgG interactions are largely ionic in nature; (2) lysine, glutamic acid and/or aspartic acid, but not arginine and tryptophan residues are in the C1q receptor site on the Cγ2 domain. Here, we have chemically modified and mapped a lysine residue which confirms the location of the postulated C1q receptor site. This is the first time direct chemical studies have led to positive identification of the site of any immunoglobulin effector function.

2. Materials and methods

2.1. *Modification of IgG*

Immune rabbit IgG (anti-ovalbumin) was prepared as in [21] then modified with [1-^{14}C]acetic anhydride which reacts with lysine residues [19,20]. The conditions of modification, acetate buffer at pH 5.0 and 0°C, are extremely mild and do not lead to protein denaturation.

Comparison of precipitin curves and circular dichroism spectra for the modified and control IgG were also as in [21].

2.2. *C1q–IgG aggregates binding assay*

Both the modified and control rabbit anti-ovalbumin IgG were formed into antigen–antibody aggregates at the same equivalence as determined from precipitin curves. The binding of ^{125}I-labelled C1q to these aggregates was measured by a modification [21] of the method in [33].

2.3. *HPLC separation*

This was performed on a Bondapak C_{18} column in 0.1% TFA/solvent X (methanol:acetonitrile:2-propanol) (1:1:1, by vol.) or in 0.1% ammonium bicarbonate/

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acetonitrile as in [34] using a Water Associates HPLC system.

2.4. *Amino acid sequence determination*

These were carried out on a Beckman 890C sequencer as detailed in [34].

3. Results

The overall strategy of correlating the functional loss of C1q binding activity with identification of the modified lysine(s) is shown in fig.1. We had shown that modification of lysines inhibits C1q–IgG complex formation [21]. The modification was minimal to ensure that there was: (1) no alteration in either conformational integrity or the antigen binding capacity of the IgG molecules measured by CD spectra and precipitin curves; (2) specific labelling of few lysine residues. The specific activity of the modifying acetate groups indicated that there was 1.2 ± 0.2 modified residues/whole IgG. After modification the functional dissociation constant of IgG and C1q was significantly altered (fig.2) in comparison with unmodified IgG immune aggregates from the same rabbit. There was no change in relative capacity of IgG for C1q. The functional dissociation constant for the modified aggregates is 2.3×10^{-8} M^{-1} and for the control aggregates is 1.0×10^{-8} M^{-1}. These data

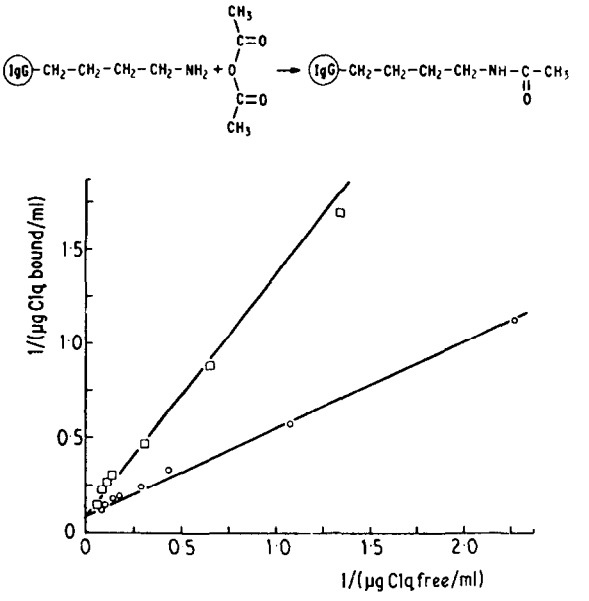

Fig.2. C1q binding to acetic anhydride modified IgG aggregates. The acetic anhydride reaction with lysine residues is shown. The inverse of the amount of C1q bound vs the inverse of the C1q free is plotted for the control aggregates (○) and the acetic anhydride modified aggregates (□). The lines computed fit to the experimental points using a non-linear least squares program. The values of K_d (and the standard error) so obtained are 1.0×10^{-8} M^{-1} (0.17) and 2.3×10^{-8} M^{-1} (0.3) for the control and modified aggregates, respectively. The corresponding relative values of the capacities are 1.0 (0.06) and 1.03 (0.07).

Flow Plan of Identification of Modified Lysines on Rabbit IgG

(1) [^{14}C] Acetic Anhydride Modification of Rabbit IgG

(2) Test of Conformational Integrity
- (a) Precipitin Curve
- (b) Circular Dichroism Spectra

(3) Test of C1q Binding - Fig. 2.

(4) Reduction and Alkylation of IgG.

(5) Separation of Heavy and Light Chains.

(6) CNBr Cleavage of Heavy Chain.

(7) Fractionation of Radiolabelled CNBr Fragments - Fig. 3a.

(8) Pepsin Digest of Radiolabelled CNBr Fragments.

(9) Isolation and Purification of Peptic Fragments - Fig. 3b.

(10) Sequence Determination of Peptic Fragments - Fig. 4a.

Fig.1.

agree with our results for acetic anhydride modified IgG where 2.2 ± 0.3 modified lysine residues on IgG changed the binding affinity 2.9-fold [21]. This is a significant change in binding affinity since modifications of arginine and tryptophan residues have no effect on C1q binding affinity for IgG.

The modified IgG was then subjected to peptide mapping to identify the modified lysine residue(s) as in fig.1. After the separation of heavy and light chains [22] it was determined by the specific activity that ~20% of the radioactive acetate groups were in the light chain. Paper electrophoresis in SDS of the heavy chain CNBr digest [23] indicated that all the radioactive acetate groups were in one peak. Fractionation of the digest using Ultrogel Aca 54 in 30% (v/v) HCOOH resolved 2 peaks. One of these contains 25% of the recovered radioactivity and from PAGE analysis probably corresponds to aggregated material, the C1 peptide (up to residue 252), and products of partial cleavage at methionine residues [37]. The main radioactive peak (75%) corresponds to the C3 peptide (residues 252–350) and a large peptide [37]. The HPLC purification of this main radioactive peak similarly indicated that the radioactive acetate groups were in one fragment (fig.3a). The whole of the main radioactive peak of CNBr fragments was digested by pepsin [24] and then purified on a G-25 gel filtration column. The radioactive peak of the gel filtration effluent was concentrated and then further purified on HPLC (fig.3b). The 2 radioactive peaks on the final HPLC column, peptide A (the minor peak) and peptide B (the major peak), were then sequenced. Peptide A was a shorter subfragment of peptide B, with the identical lysine residue modified. The 2 fractions containing peptides A and B represent 8% and 44% of the radioactivity applied to the column and they constitute 70% of the total recovered radioactivity.

The sequence determination of peptide B gave a sequence in the Cγ2 domain between residues 319 and 332 (fig.4a). The peptide A sequence was between residues 319 and 322. In the peptide B sequence there are 3 lysine residues: Lys (320), Lys (322) and Lys (326). Only Lys (322) was modified. The quantitative yield of Lys (322) in the sequence determination revealed that only 80% was modified. This shows that the HPLC purification does not preferentially distinguish the modified and unmodified peptides containing this lysine. The specific activity of this acetyl lysine residue was 8×10^3 dpm/nm compared to a specific activity of $\sim 2.5 \times 10^3$ dpm/nm for the entire heavy chain. Neither Lys (320) nor Lys (326) were modified to any detectable extent, <1% of Lys (322).

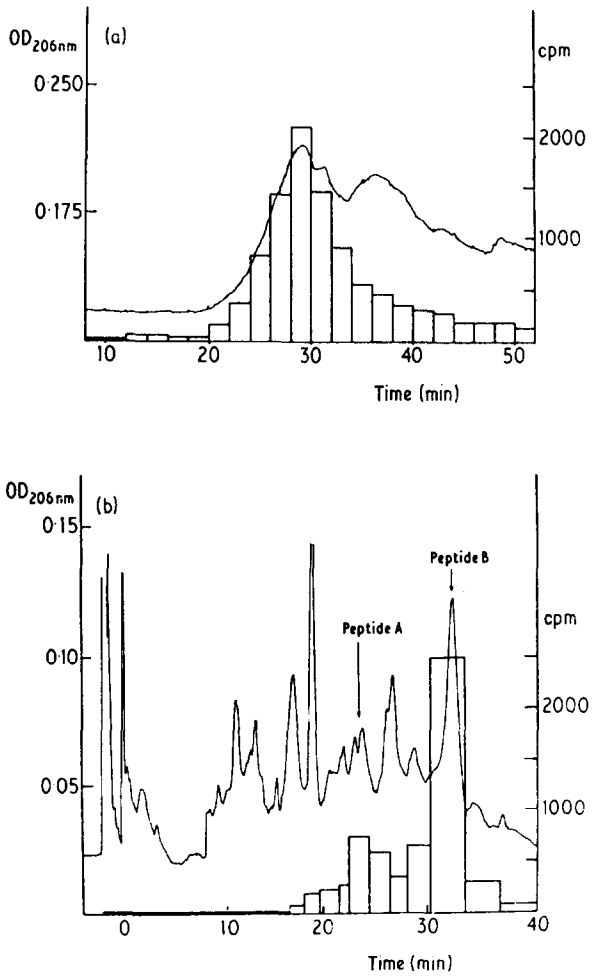

Fig.3. HPLC on CNBr fragments and peptic fragments. The chromatography was performed at 1 ml/min flow rate. In (3a) the CNBr fragments of the H-chain were chromatographed using the system methanol:acetonitrile:2-propanol (1:1:1, by vol). In (3b) the peptic fragments of the main radioactive peak (75%) of CNBr products was applied to the column using the 0.1% ammonium bicarbonate acetonitrile system. In both (3a) and (3b) time is in minutes after the beginning of the gradient. The absorbance was monitored at 206 nm. The radioactivity measurements in the histogram were performed on 500 l samples from (a) and 200 l samples from (b) in 0.5% 2,5-diphenyloxazole in dioxan scintillant. From the second HPLC run (3b) peptide A and peptide B were isolated and sequenced.

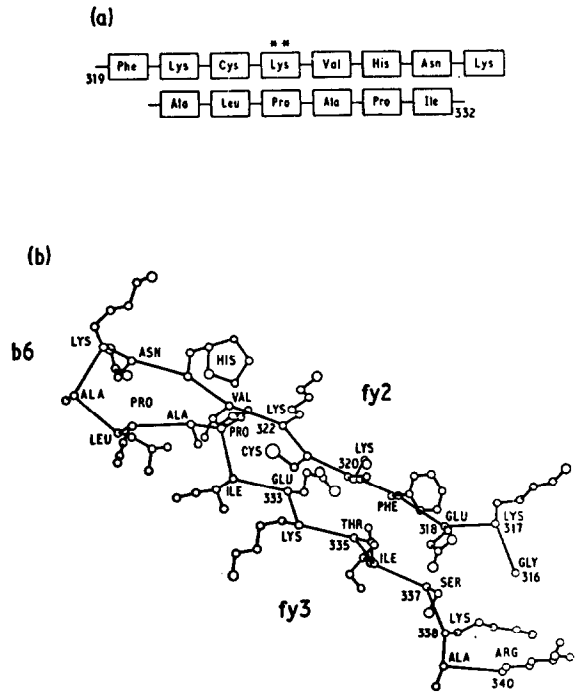

Fig.4. The sequence and conformation of the C1q binding site on the Cγ2 domain of IgG. (a) The primary sequence of peptide B isolated from the peptic fragments using HPLC (fig.3b). The radiolabelled acetyl lysine is indicated (**). (b) The Cγ2 domain containing peptide B (residues 319–322) and the remaining portions of the fy2 and fy3 β-strands from rabbit IgG are shown. This view is derived from the crystallographic data in [7] for human Fc using the amino acid side chains for rabbit IgG.

4. Discussion

The specific modification of Lys (322) with the loss of C1q binding affinity clearly demonstrates that this lysine is in or near to the C1q receptor site on IgG. This verifies our original postulate that residues in the 2 C-terminal β-pleated sheets of the Cγ2 domain, which contains Lys (322) (fig.4b), are the C1q receptor site.

The modification of Lys (322) does not delineate the extent of the C1q receptor site on IgG. However, several other residues of the fy2 and fy3 β-strands (nomenclature from [25]) are probably necessary for a C1q receptor site on IgG. A likely set of conserved, accessible residues consistent with our chemical modification studies and continuous with Lys (322) that could serve as a C1q receptor site are: Glu (318), Lys (320), Lys (322), Glu (333), Thr (335) and Ser (337). These are all part of the 2 parallel β-sheets, fy2 and fy3. The b6 bend which links these 2 parallel β-sheets is probably not part of the C1q binding site since it is involved in Cγ1–Cγ2 domain interactions [7] (fig.4b).

We had demonstrated that C1q–IgG complex formation is largely the result of ionic interactions and is not driven wholly by shielding of hydrophobic residues as proposed [26]. The C1q receptor site identified here is consistent with this conclusion. All of these residues can participate in ion bridges either between oppositely charged residues or between similarly charged residues using bridging water or chloride molecules [27]. Since the binding between C1q and monomer IgG is not very tight, with a dissociation constant of 10^{-4} M^{-1}, it is conceivable that several ionic bridges may comprise the total binding energy of the C1q–IgG interaction. Consistent with this idea, modification of the single Lys (322) with a loss of its charge (fig.2) causes a moderate decrease in the binding affinity of C1q for IgG but does not eliminate binding, which would be reflected in a decrease in the capacity of IgG for C1q.

The C1q–IgG binding described here contrasts with that for antigen–antibody systems. The antigen binding site on antibodies involves a cleft or a groove constructed from 2 distinct chains into which the antigen fits. The antigen binding to antibodies is a shape problem in which the different antigens alter their position within the combining site so as to maximize Van der Waal's and hydrophobic interactions [28,29]. The C1q binding to antibodies on the other hand is a surface matching problem. The C1q receptor site on IgG is a β-strand array of charged and polar residues and recognition probably requires a complementary array of charged and polar residues on the C1q molecule. This type of surface contact may be the main type of interaction in the recognition between large proteins [30,31]. Cytochrome *c* peroxidase–cytochrome *c* recognition may occur by a matching of charged arrays containing lysine and aspartic acid residues [32]. The other immunoglobulin effector functions may be mediated by a similar surface-charged array to that for C1q binding on IgG. Localization of the sites of these effector functions on the immunoglobulin Fc utilizing an approach similar to that outlined in [18] and here, by our identification of the C1q receptor site, should test this postulate.

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